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Intracellular pH regulation in U-2 OS human osteosarcoma cells transfected with P-glycoprotein

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Abstract

The molecular mechanisms responsible for intracellular pH regulation in the U2-OS osteosarcoma cell line were investigated by loading with 2′,7′-bis(2-carboxyethyl)-5(6) carboxyfluorescein ester and manipulation of Cl^- and Na^+ gradients, both in HEPES- and HCO_3^-/CO_2-buffered media. Both acidification and alkalinisation were poorly sensitive to 4,4′-diisothiocyanate dihydrostilbene-2,2′-disulfonic acid, inhibitor of the anion exchanger, but sensitive to amiloride, inhibitor of the Na^+/H^+ exchanger. In addition to the amiloride-sensitive Na^+/H^+ exchanger, another H^+ extruding mechanism was detected in U-2 OS cells, the Na^+-dependent HCO_3^-/Cl^- exchanger. No significant difference in resting pH_i and in the rate of acidification or alkalinisation was observed in clones obtained from U-2 OS cells by transfection with the *MDR1* gene and overexpressing P-glycoprotein. However, both V_{max} and K' values for intracellular $[H^+]$ of the Na^+/H^+ exchanger were significantly reduced in MDR1-transfected clones, in the absence and/or presence of drug selection, in comparison to vector-transfected or parental cell line. NHE1, NHE5 and at a lower extent NHE2 mRNA were detected in similar amount in all U2-OS clones. It is concluded that, although overexpression of P-glycoprotein did not impair pH_i regulation in U-2 OS cells, the kinetic parameters of the Na^+/H^+ exchanger were altered, suggesting a functional relationship between the two membrane proteins.

Keywords: P-glycoprotein; MDR1 gene; Intracellular pH; Na^+/H^+ exchanger; Human osteosarcoma cell

1. Introduction

P-Glycoprotein is a 170 kDa membrane protein encoded by the multidrug resistance *MDR1* gene, which has a central role in the multidrug type of resistance (MDR) to anticancer agents (for a review see [1]). In vitro, P-glycoprotein is expressed in tumour cell lines in response to a number of structurally and functionally unrelated anticancer agents. This glycoprotein is believed to act as an ATP-driven transporter, that pumps toxic substances, including chemotherapeutic drugs, out of drug-resistant tumour cells [1,2]. However, other studies, focused on the kinetic and thermodynamic details of drug translocation in cells expressing P-glycoprotein, have suggested that the model of drug pump is inadequate

Abbreviations: pH_i, intracellular pH; BCECF, 2′,7′-bis(2-carboxyethyl)-5(6) carboxyfluorescein ester; H_2DIDS, 4,4′-diisothiocyanate dihydrostilbene-2,2′-disulfonic acid; MDR, multidrug resistance; β_i, intracellular buffering capacity; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

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[3,4] and have stimulated the proposal of alternative models for P-glycoprotein-mediated drug resistance. According to one of these alternative models, P-glycoprotein overexpression would not directly cause the efflux of drugs out of cells, but would alter cellular retention of anticancer drugs *indirectly*, by modulating intracellular pH (pH_i), volume, and/or plasma membrane potential. Significant changes in one or more of these parameters were found in cells overexpressing P-glycoprotein [5,6]. Most toxic drugs are weakly basic, positively charged at physiological pH. An increase in pH_i or a decrease of negative internal electrical membrane potential would reduce intracellular retention of these cationic compounds.

This model becomes even more relevant, considering that overexpression of the cystic fibrosis transmembrane conductance regulator (CFTR), another member of the ABC family of transporters, has also been shown to perturb pH_i and membrane potential regulation [7–9]. According to Hoffman and Roepe [10], there are two crucial aspects which make reliable studies on regulation of cytoplasmic pH in P-glycoprotein expressing cells: (i) the use of true transfectants, i.e., cell lines not exposed in any way to chemotherapeutic drugs prior to analysis, to avoid alterations of P-glycoprotein-mediated phenotype, introduced by exposing cells to drugs; (ii) pH_i has to be determined in media buffered with HCO_3^-/CO_2, in order to examine pH_i regulatory processes under close-to-physiological conditions. Under these conditions, it has been reported that short-term isotonic substitution of Cl^- in the incubation medium induces cytoplasmic acidification, which is independent of HCO_3^-/CO_2 or Na^+, since it occurs also in HEPES-buffered media or in media where Na^+ is replaced by K^+. This acidification is partially inhibited by verapamil, suggesting that a novel ATP and Na^+-dependent Cl^-/H^+ antiport process might be directly or indirectly mediated by P-glycoprotein [10].

We have recently obtained P-glycoprotein overexpressing cell clones of U-2 OS human osteosarcoma cell line by co-transfection with *MDR1* gene and *neo* gene, generating two clones, U-2/MDR117.1 and U-2/MDR117.2. Another clone was obtained by transfection with *MDR1* gene and further selection in the presence of the anticancer drug doxorubicin (U-2/DOXO-35 clone) [11]. Interestingly, transfection of U-2 OS osteosarcoma cells with MDR1 gene and the consequent overexpression of P-glycoprotein on their plasma membrane were shown to significantly impair their malignant potential, both in vitro and in vivo, indicating the existence of a cause-and-effect relationship between the expression of P-glycoprotein and the reduced malignancy of osteosarcoma MDR cells [11]. Indeed, reduction of the tumourigenic and metastatic ability of cancer cells appears to be a constant feature of MDR in many, although not in all, the experimental models [12]. The mechanisms triggering the loss of malignancy of MDR cells are still completely unknown, raising new questions on the function of P-glycoprotein under physiological conditions. The recent evidence indicating that P-glycoprotein can act as an ion transport regulator prompted us to investigate whether overexpression of P-glycoprotein in U-2 OS cells causes a perturbation of resting value of pH_i, as the result of operation of a Cl^--dependent H^+ flux, directly or indirectly mediated by P-glycoprotein, as suggested in [10]. Furthermore, we determined the molecular mechanisms responsible for pH_i regulation in U-2 OS clones, by manipulation of Cl^- and Na^+ gradients, both in HEPES- and HCO_3^-/CO_2-buffered media. We failed to detect any significant change in the resting pH_i values and in the rate and extent of both acidification and alkalinisation, among the parental cell line U-2 OS and two clones expressing P-glycoprotein, both in the presence or absence of selection with doxorubicin. However, two H^+ extruding mechanisms were identified in U-2 OS cells, the Na^+/H^+ exchanger and the Na^+-dependent HCO_3^-/Cl^- exchanger. The rate of H^+ efflux through the Na^+/H^+ antiporter was significantly reduced in clones overexpressing P-glycoprotein, in comparison with parental U-2 OS cells or vector-transfected cells (U-2/neo8), whereas H^+ efflux through the Na^+-dependent HCO_3^-/Cl^- exchanger was not modified.

2. Materials and methods

2.1. Materials

2′,7′-bis(2-carboxyethyl)-5,6-carboxyfluorescein tetraacetoxymethyl ester (BCECF) and 4,4′-diisothiocyanate dihydrostilbene-2,2′-disulfonic acid (H_2DIDS) were purchased from Molecular Probes (Eugene, OR); amiloride, bafilomycin A, doxorubi-

cin, geneticin (G418) and nigericin were from Sigma (St. Louis, MO).

2.2. Cell culture and transfection

U-2 OS human osteosarcoma cells were maintained in Iscove's modified Dulbecco's medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% foetal calf serum (FCS) at 37°C in an humidified 5% CO_2 atmosphere. Cells were transfected with 10 μg pf pFR-CMV, an expression vector containing a full-length *MDR1* cDNA kindly provided by Dr P. Borst (Division of Molecular Biology, the Netherlands Cancer Institute, Amsterdam, The Netherlands). After 24 h exposure to DNA, cells were maintained in normal medium for 2 more days. Cultures were then exposed to selective medium containing 300 ng/ml of doxorubicin and refed every other day until selection of resistant colonies, after 2 weeks. Using glass cloning cylinders, several transfected clones were obtained. U-2/DOXO-35 is representative of this series. U-2 OS cells were also co-transfected with 10 μg/ml pf pFR-CMV and 1 μg of pSV2*neo*, an expression vector containing the neomycin resistance *neo* gene. These clones were selected in medium containing 500 mg/ml of the neomycin analogue G418. U-2/MDR117.1 and U-2/MDR117.2 clones are representative of this series. Controls were obtained by transfection with calf serum thymus DNA and pSV2*neo*, and selection with G418 (500 μg/ml). U-2/neo8 is representative of this series. In parallel with pH_i determination experiments, P-glycoprotein expression at the cell membrane of the different cell lines was routinely analysed by indirect immunofluorescence and flow cytometry, as described in [11].

2.3. Intracellular pH measurements

Trypsinised cells (4×10^6 cells/ml) were incubated for 30 min with a saline solution containing 135 mM NaCl, 3 mM KCl, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 10 mM D-glucose, 1 mM $CaCl_2$ and 10 mM Na-HEPES, pH 7.4, and 4 μM 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein tetraacetoxymethyl ester (BCECF-AM) at 37°C. BCECF leakage from the cells was negligible. BCECF fluorescence was measured in the cuvette compartment (37°C, with magnetic stirring) of a Jasko SP-770 Spectrofluorimeter (Tokyo, Japan), with excitation and emission wavelengths of 505 and 530 nm, respectively. Aliquots of BCECF loaded cells (3×10^5 cells) were suspended in different solutions, as detailed subsequently, and transferred in a cuvette plugged with a snugly fitted teflon holder. The holder had inlet and outlet ports to permit continuous gassing of cellular suspension with a 95% O_2/5%CO_2 mixture. Before use, the saline solutions containing 25 mM $NaHCO_3$ were bubbled with the 95% O_2/5%CO_2 mixture for several minutes and their pH, determined before and after cell fluorescence recordings, was 7.4. In some cases, experiments were performed in the absence of HCO_3^-/CO_2, 10 mM HEPES replacing HCO_3^-. Calibration curves of pH_i against fluorescence were generated in cells incubated with 1 μM nigericin in high K^+ medium. Fluorescence was linear with pH over the range from pH 6.5 to 8.0; the coefficient of linear regression was higher that 0.95 in all the experiments. The rate of change in pH_i (ΔpH_i/min) was measured by linear regression of traces within the first minute. The extent of pH_i change was determined as the difference between the initial value and that measured at steady state.

2.4. Determination of net acid extrusion

BCECF-loaded cells were incubated in Na^+-free N-methyl-D-glucamine (NMG^+) solution (pH 7.4) and pH_i was clamped at the desired values by addition of 0.6 μM nigericin and 4.5 mg/ml BSA, as described in [13], then 40 mM NaCl was added. Where indicated, 0.5 mM amiloride was added before NaCl, followed by 20 mM $NaHCO_3$. Alternatively, acidification was induced by preincubation at room temperature with 10 mM NH_4Cl for different times and dilution in Na^+-free NMG-Cl containing saline solution as described in [14]. H^+ efflux rates (μM H^+/s), equivalent to the rate of Na^+/H^+ exchange, were then determined by multiplying the rate of alkalinisation by the cellular buffering capacity at the corresponding pH value. Scatter plots of H^+ efflux rate versus intracellular [H^+] were constructed using data from four or more separate experiments, over the range of pH_i of 6.2–7.3. Kinetic analysis did not include data from cells with intracellular pH lower then 6.2, which was the lowest value that could be reached. The Na^+/H^+ exchange rate data were analysed using a nonlinear least-

squares minimisation routine based on a modified Marquardt algorithm [15], which allowed fitting of data to a general allosteric model described by the Hill equation ($v = V_{max}*[S]^n/K'+[S]^n$) with estimates for V_{max} and K' and Hill coefficients (n_{app}) [16].

2.5. Determination of intracellular buffering power

Intracellular buffering power (β_i) was determined by incubating cells in Na^+-free NMG-Cl solution, and clamping intracellular pH at a desired value by addition of nigericin/BSA, as described above, then adding 5 mM NH_4^+ and determining the pH_i increase. β_i ($\Delta[NH_4^+]/\Delta pH_i$) was calculated for each cell line clamped at different pH_i, as described in [17].

2.6. Semi-quantitative reverse transcriptase–polymerase chain reaction (RT–PCR) for NHE isoforms

Total RNA was isolated from frozen cell pellets using TRIzol extraction kit (Life Technologies, Paisley, UK). Reverse transcription was performed using M-MLV (Life Technologies) in the presence of oligo-dT and dNTP. The RT–PCR exponential phase was determined on 20–35 cycles to allow a semi-quantitative comparison among the cDNAs developed from identical reactions. Specific primer pairs for NHE1, 2, 3 were designed as reported in [18]. Human NHE5 primers were designed from the cDNA sequence for this isoform [19]: 5′ primer, 5′-GTGTTTCACCTGTCTCGGAAAG-3′ and 3′ primer, 5′-GATGGCACCCAAGTTGTCAAAG-3′, length of amplified region 219 residues; nucleotides 256–474 of the human NHE5, U08607 [20]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer pairs were obtained from Clontech (Palo Alto, CA; 983 bp product). The PCR cycling conditions were 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s using GeneAmp PCR System 9700 (Perkin Elmer, Norwalk, CT). A negative control was used to rule out any contamination due to genomic DNA, with RT reactions run in the absence of the RT enzyme followed by PCR. Amplified products were identified by 2% agarose gel electrophoresis after staining with ethidium bromide. The Gel Documentation system (Bio-Rad, Hercules, CA) was used for quantification of the results. The intensity of PCR products, at 28 cycles, was normalised on the basis of the intensity of GAPDH PCR product.

2.7. Solutions

HCO_3^--buffered, Cl^--free solution contained 115 mM Na-gluconate (or Na-glutamate, we did not observe significant difference between experiments performed with the different impermeant anions), 3 mM KCl, 2 mM KH_2PO_4, 1 mM $MgSO_4$, 10 mM D-glucose, 1 mM Ca-gluconate and 25 mM $NaHCO_3$ (pH 7.4). Some experiments were carried out in the absence of HCO_3^- (10 mM HEPES instead of HCO_3^-) or Na^+ (equimolar NMG^+ or K^+ instead of Na^+) or Na^+ and Cl^- (equimolar NMG^+- or K^+-glutamate). In HEPES-buffered solutions the concentration of the main salt was 135 mM. Osmolality of solutions was 280–295 mosmol/kg.

2.8. Statistical evaluation

All values are expressed as mean ± standard deviation (S.D.), with the number of experiments in brackets; P is the level of significance on a Student's t-test.

3. Results

3.1. Resting pH_i and intracellular buffering capacity of various U-2 OS clones

Table 1 reports the values of pH_i determined in the various U-2 OS cell lines, both in HEPES- and HCO_3^--buffered saline solutions. It is apparent that

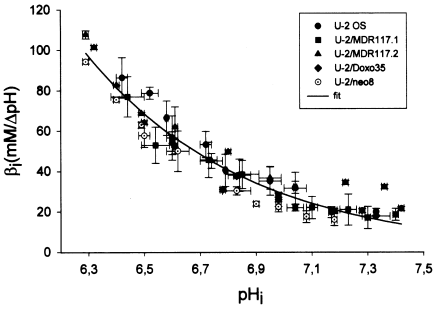

Fig. 1. Intracellular buffering power (β_i) as a function of pH_i in different U-2 OS clones. BCECF-loaded cells were incubated in Na^+-free NMG-Cl saline solution in the presence of 0.6 μM nigericin and clamped at different pH_i by addition of 4.5 mg/ml BSA. Five mM NH_4Cl was added and the initial increase of pH_i due to influx of NH_3 was used for calculating β_i. Each data point is mean ± S.D. of at least three determinations. The fit has been determined from data obtained in all clones, since it was superimposed to that determined on each cell line.

steady-state pH_i values of the various cell lines, incubated in HEPES-buffered media were significantly higher than pH_i in HCO_3^--buffered media, as previously described in [14], and that overexpression of P-glycoprotein did not modify the values of steady-state pH_i. In fact, no significant variation was observed between MDR transfectants (either U-2/MDR117.1 and U-2/MDR117.2 and in U-2/DOXO-35 selected in doxorubicin) and the parental cell line U-2 OS or vector-transfected U-2/neo8 cells.

Fig. 1 shows that the values of the intracellular buffering power (β_i) were strongly dependent on pH_i, gradually increasing with the decrease of pH_i, and were similar in the various U-2 OS cell lines.

3.2. Intracellular acidification in U-2 OS clones incubated in HEPES-buffered media

To assess whether P-glycoprotein overexpression caused a perturbation of H^+ fluxes, experiments with cells incubated in Cl^--free, HEPES-buffered solutions were carried out. Hoffman and Roepe, 1997, indeed reported that rapid switch from HEPES-buffered NaCl to HEPES-buffered Na-gluconate solution caused the occurrence of a dramatic acidification [10]. Fig. 2A illustrates the results obtained with parental U-2 OS cells kept in HEPES-buffered NaCl solution and then incubated in equimolar Na-gluconate (similar results were obtained with glutamate, not shown). A weak cytosolic acidification was observed, which was not affected by the addition of the well-known anion exchanger inhibitor H_2DIDS (not shown), in agreement with previously reported data in rat pancreatic acinar cells [21]. Substitution of extracellular Na^+ with NMG^+ in the presence of Cl^-, significantly increased both the rate and the extent of acidification, this latter being almost completely blocked by the addition of 0.5 mM amiloride, an inhibitor of the Na^+/H^+ exchanger (Fig. 2A). This finding indicates that in the absence of extracellular Na^+, Na^+ can exit from the cells according to its chemical gradient through the reversal of the Na^+/H^+ antiporter. When both Na^+ and Cl^- were replaced by equimolar K^+ (or NMG^+, not shown) and gluconate (or glutamate, not shown), the rate of pH_i decrease was further increased (Fig. 2B, and also Table 2). This acidification was poorly sensitive to H_2DIDS (not shown) and still sensitive to amiloride, suggesting the involvement of Na^+/H^+ exchanger. These findings are in agreement with previously reported data showing that in HEPES-buffered media, the Na^+/H^+ exchanger is the only operative mechanism used to regulate pH_i [21]. Preincubation with bafilomycin A, an inhibitor of the vacuolar ATPase, failed to significantly modify the rate of acidification in HEPES-buffered Na-gluconate medium either in the absence or presence of amiloride (results not shown).

Table 2 reports that the values of the rate and extent of acidification of parental U-2 OS cells, of the clones expressing P-glycoprotein and of U-2/DOXO-35 cells were not significantly different.

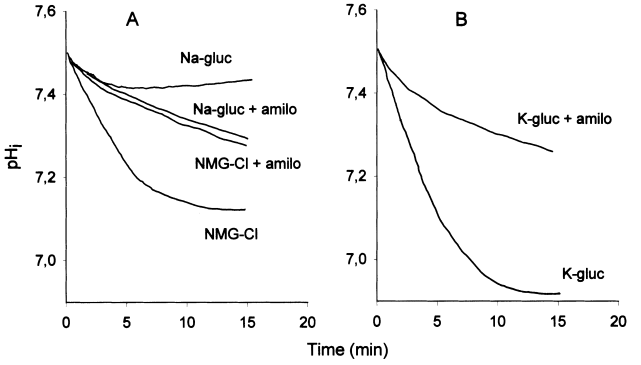

Fig. 2. Changes of pH_i in U-2 OS cells incubated in HEPES-buffered Cl^-- and Na^+-free media. BCECF-loaded cells were incubated in HEPES-buffered, Cl^--free Na-gluconate medium or Na^+-free NMG-Cl medium (A) or Cl^-/Na^+-free K-gluconate medium (B). Where indicated, 0.5 mM amiloride (amilo) was added. Data are representative of four highly consistent experiments.

Table 1
Resting pH_i values in different U-2 OS clones

	U-2 OS	U-2/neo8	U-2/MDR117.1	U-2/MDR117.2	U-2/DOXO-35
HEPES medium	7.44 ± 0.07 (31)	7.47 ± 0.12 (40)	7.49 ± 0.08 (30)	7.40 ± 0.1 (21)	7.45 ± 0.23 (32)
HCO_3^- medium	7.03 ± 0.07 (15)	7.03 ± 0.04 (9)	7.05 ± 0.12 (23)	7.02 ± 0.11 (20)	7.12 ± 0.28 (20)

Values are mean ± S.D. For determination of pH_i values in cells incubated in HCO_3^--buffered NaCl saline solution, cells were loaded with BCECF/AM in HCO_3^--buffered saline solution.

Table 2
Rate and extent of pH_i decrease of different U-2 OS clones incubated in Cl^--free or Na^+-free or Na^+/Cl^--free HEPES-buffered media

	U-2 OS	U-2/MDR117.1	U-2/MDR117.2	U-2/DOXO-35
	Rate of acidification (ΔpH/min)			
Na-glucon medium	0.044 ± 0.03 (5)	0.042 ± 0.013 (4)	0.030 ± 0.01 (3)	0.034 ± 0.011 (3)
NMG-Cl medium	0.048 ± 0.015 (3)	0.051 ± 0.021 (3)	0.054 ± 0.03 (4)	0.084 ± 0.046 (4)
NMG-glutam medium	0.112 ± 0.016 (2)	0.086 ± 0.001 (2)	0.095 ± 0.03 (2)	0.180 ± 0.059 (4)
K-glucon medium	0.123 ± 0.012 (2)	0.088 ± 0.018 (2)	0.095 ± 0.04 (2)	0.142 ± 0.028 (4)
	Extent of acidification (ΔpH)			
Na-glucon medium	0.12 ± 0.11 (5)	0.18 ± 0.08 (3)	0.12 ± 0.01 (2)	0.15 ± 0.03 (3)
NMG-Cl medium	0.33 ± 0.08 (3)	0.34 ± 0.12 (3)	0.43 ± 0.14 (4)	0.30 ± 0.02 (4)
NMG-glutam medium	0.96 ± 0.03 (2)	0.54 ± 0.07 (2)	0.66 ± 0.30 (2)	0.60 ± 0.18 (4)
K-glucon medium	0.83 ± 0.03 (2)	0.47 ± 0.07 (2)	0.60 ± 0.12 (2)	0.67 ± 0.07 (4)

The rate and extent of acidification were determined as described in Section 2.

3.3. *Intracellular alkalinisation in U2 OS clones incubated in HCO_3^--buffered media*

The experiments reported in Fig. 3 were performed in U-2 OS cells incubated with Cl^--free, HCO_3^--buffered media equilibrated with 5% CO_2. The value of pH_i increased from approximately 7.0 to a new steady-state value of 7.5. Under these conditions, the concentration-driven Cl^- efflux from cells should promote a fast intracellular alkalinisation due to stoichiometric HCO_3^- influx. However, the addition of 0.15 mM H_2DIDS only slightly reduced the rate, but not the extent, of alkalinisation, suggesting that the activities of the HCO_3^-/Cl^- exchange and of the Na^+/HCO_3^- cotransport, both inhibited by H_2DIDS, were not involved.

To test the presence in U-2 OS of HCO_3^-/Cl^- exchanger activity, cells were incubated with Na-gluconate saline solution at pH 8.0, in the presence of 0.6 μM nigericin, as described in [6]. Under this condition, the pH_i values were 7.8–7.9. It is well known that the activity of the anion exchanger is maximal at these elevated pH_i values [22]. The subsequent addition of Cl^- failed to induce any significant acidification (result not shown). As an alternative approach, after loading with BCECF, cells were maintained in Cl^--free, HCO_3^--buffered Na-gluconate saline for 30 min, then centrifuged and incubated in HEPES-buffered NaCl saline solution. Under these conditions, Cl^- influx would drive HCO_3^- efflux from cells. Indeed, a weak acidification was observed, which however was completely unaffected by H_2DIDS (not shown). These findings support the notion that the parental U-2 OS cell line lacks a significant Na^+-independent HCO_3^-/Cl^- exchanger activity.

As reported in Fig. 3A, pH_i increase was strongly

Table 3
Rate and extent of pH_i increase due to incubation in Cl^--free Na-glutamate, HCO_3^--buffered medium

	U-2 OS	U-2/MDR117.1	U-2/MDR117.2	U-2/DOXO-35
	Rate of acidification (ΔpH/min)			
Control	0.081 ± 0.003* (3)	0.090 ± 0.009 (3)	0.088 ± 0.050 (5)	0.184 ± 0.088* (5)
0.15 mM H_2DIDS	0.060 ± 0.019 (2)	0.044 ± 0.017 (2)	0.052 ± 0.012 (3)	0.116 ± 0.070 (2)
0.5 mM amiloride	0.035 ± 0.039* (2)	0.066 ± 0.030 (3)	0.021 ± 0.006 (2)	0.137 ± 0.033* (4)
H_2DIDS+amiloride	0.030 ± 0.003 (2)	0.017 ± 0.020 (4)	0.022 ± 0.013 (5)	0.057 ± 0.021 (5)
	Extent of acidification (ΔpH)			
Control	0.42 ± 0.10 (3)	0.36 ± 0.05 (3)	0.36 ± 0.09 (5)	0.64 ± 0.11 (5)
0.15 mM H_2DIDS	0.42 ± 0.09 (2)	0.39 ± 0.09 (2)	0.37 ± 0.02 (3)	0.55 ± 0.02 (2)
0.5 mM amiloride	0.06 ± 0.02 (2)	0.12 ± 0.07 (2)	0.12 ± 0.01 (2)	0.12 ± 0.01 (4)
H_2DIDS+amiloride	0.01 ± 0.01 (2)	0.04 ± 0.03 (4)	0.04 ± 0.02 (5)	0.03 ± 0.03 (5)

The rate and extent of alkalinization were determined as described in Section 2. In each column, * denotes values significantly different, $P < 0.05$.

reduced by addition of 0.5 mM amiloride and completely abolished by addition of both amiloride and H_2DIDS, indicating that also in HCO_3^--buffered saline solutions Na^+/H^+ exchanger seems to be the main transport mechanism responsible for pH_i regulation.

Removal of Na^+ and substitution with K^+ caused a sudden acidification, which was followed by a slow alkalinisation (Fig. 3B). In the presence of amiloride, acidification by K-gluconate was completely reversed and traces were superimposable on those obtained in cells exposed in Na-gluconate medium in the presence of the inhibitor. To exclude that Na^+ substitution with K^+ might alter pH_i by collapsing the plasma membrane potential, cells were incubated with a HCO_3^--buffered saline solution containing NMG-glutamate and the results were similar to those obtained with K-glutamate (not shown). These findings confirm that the alkalinisation induced by Cl^--free, HCO_3^--buffered media is mainly due to the activity of the Na^+/H^+ exchanger.

In Table 3, the values of the rate and extent of alkalinisation in the various U-2 clones are shown. It is noteworthy that basal rates of alkalinisation were similar in all U-2 OS clones.

3.4. Na^+-dependent acid extrusion mechanisms

Fig. 4A illustrates that the removal of extracellular Na^+ and substitution with NMG-Cl saline solution elicited a pH_i decrease of U-2 OS cells, which was further enhanced by the addition of the K^+/H^+ ionophore nigericin (0.6 μM). Acidification was clamped at a defined pH_i by addition of excess BSA (4.5 mg/ml), as described in [6,13]. Further addition of NaCl promoted the rapid recovery of pH_i, which was completely abolished by 0.5 mM amiloride (Fig. 4A). At pH_i 6.6, the initial rate of pH_i recovery was 0.504 ± 0.055 ΔpH/min, $n = 9$, and was dependent on the extracellular concentration of Na^+ (Fig. 4B). By linearisation of value of H^+ flux according to Lineweaver and Burk, a straight line with a significant correlation coefficient (0.97) was obtained. From this plot, the maximal rate of Na^+/H^+ exchanger was found to be 35.9 μM/min and half-maximal stimulation was attained at 45 mM $[Na^+]_{out}$. In Fig. 4C, the dose–response curve for amiloride inhibition of the initial rate of H^+ efflux

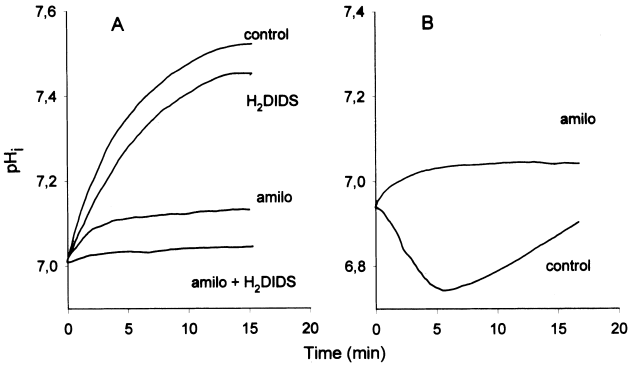

Fig. 3. Rise in pH_i of U-2 OS cells incubated in HCO_3^--buffered Cl^--free media. BCECF-loaded cells were incubated with HCO_3^--buffered Cl^--free media containing Na-gluconate (A) or HCO_3^--buffered Cl^-/Na^+-free media containing K-gluconate (B). Where indicated: 0.5 mM amiloride (amilo) and/or 0.15 mM H_2DIDS were added. Data are representative of four highly consistent experiments.

is reported, showing that the K_i for amiloride was 2 μM.

Fig. 5A shows that addition of 20 mM $NaHCO_3$ to cells clamped at pH_i 6.6 by nigericin and BSA, and treated with amiloride and NaCl, still induced a significant pH_i increase, which was almost completely inhibited by H_2DIDS concentrations higher than 50 μM. The initial rate of pH_i increase was 0.083 ± 0.030 ΔpH/min, $n = 5$. Furthermore, as illustrated in Fig. 5B, pH_i recovery was prevented by depletion of intracellular Cl^-, obtained by incubation of cells in Na^+- and Cl^--free saline solution containing NMG-glutamate. In an alternative protocol, cells were acidified to the pH_i value of 6.6 by a 4-min pre-pulse with 10 mM NH_4Cl, as described in [23], centrifugation and resuspension in a Na^+-free, NMG-Cl containing saline solution. This protocol was chosen to rule out any possible interference of the ionophore nigericin on H^+ fluxes. Addition of 40 mM NaCl gave a cytosolic alkalinisation which was superimposed to that reported in Fig. 5A (not shown). Furthermore, in the absence of nigericin, the effect of substitution of extracellular Na^+ with equimolar K^+ could be determined. When cells were treated with 0.5 mM amiloride, KCl and $KHCO_3$, no significant alkalinisation was observed (Fig. 5C). All together, the results reported in Figs. 4 and 5 indicate that the main mechanism of pH_i recovery from an acid load is the Na^+/H^+ exchanger, but also a Na^+-dependent mechanism exchanging

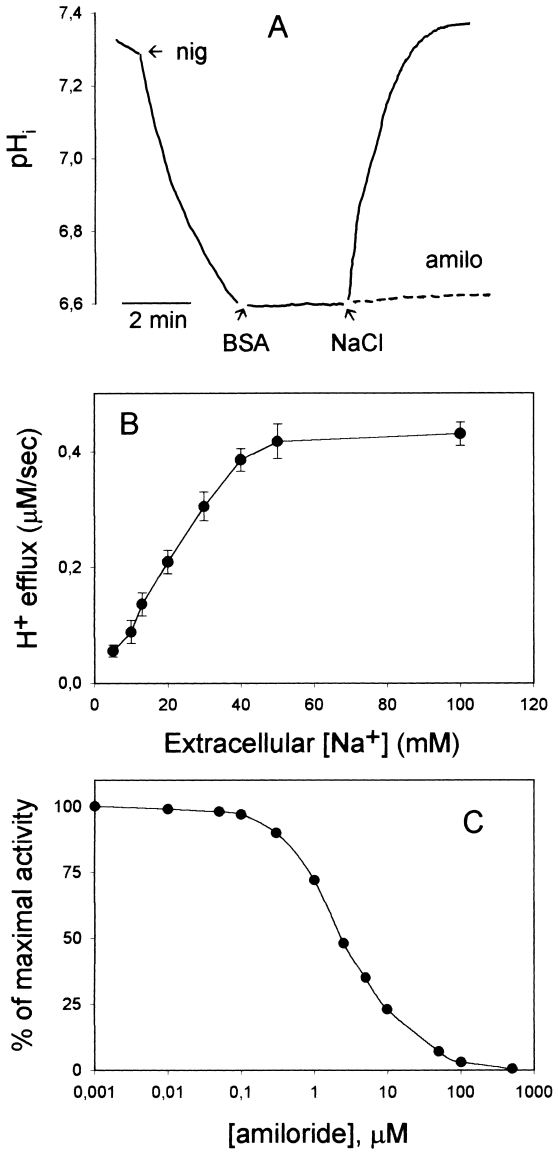

Fig. 4. Acid extrusion by the Na^+/H^+ exchanger in U-2 OS cells. BCECF-loaded cells were incubated in Na^+-free NMG-Cl saline solution. (A) At the arrows: 0.6 μM nigericin (nig), 4.5 mg/ml BSA, 40 mM NaCl, were added (continuous line); where indicated, 0.5 mM amiloride (amilo) was added before NaCl (dotted line). (B) Cells were treated as in A, clamped at pH_i 6.6 and alkalinization was started by addition of different concentrations of NaCl. The H^+ efflux rate was calculated from the initial rate of ΔpH_i/min, using the value of 57 mM/ΔpH for β_i. (C) Dose–response curve for amiloride inhibition of the rate of H^+ efflux measured as described above, at pH_i of 6.6 and NaCl 40 mM. Traces are representative of 3–5 highly consistent experiments.

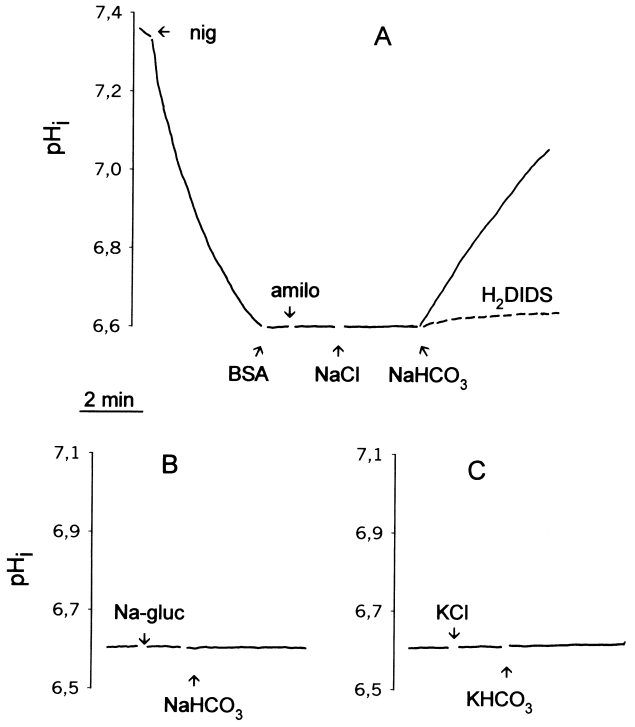

Fig. 5. Acid extrusion by the Na^+-dependent HCO_3^-/Cl^- exchanger in U-2 OS cells. (A) Cells were treated with nigericin and BSA as in Fig. 4A; where indicated, 0.5 mM amiloride, 40 mM NaCl and 20 mM $NaHCO_3$ (continuous line); 0.15 mM H_2DIDS was added before 20 mM $NaHCO_3$ (dotted line). (B) Cells were treated as in trace A, except that the saline solution contained NMG-glutamate instead of NMG-Cl, and 40 mM Na-gluconate (Na-gluc) was added instead of NaCl. (C) Cells were acidified by a 4-min pre-pulse with 10 mM NH_4Cl, then centrifuged and incubated in Na^+-free NMG-Cl saline solution containing 0.5 mM amiloride. Where indicated, 40 mM KCl and 20 mM $KHCO_3$ were added. Traces are representative of five highly consistent experiments.

extracellular HCO_3^- for intracellular Cl^- operates in U-2 OS cells.

By following the protocol described in Fig. 4A and 5A, respectively, the rates of H^+ efflux through the Na^+/H^+ and Na^+-dependent HCO_3^-/Cl^- exchangers were determined in U-2 OS cells as a function of cytoplasmic pH, and the results are shown in Fig. 6. At constant extracellular Na^+ concentration and pH, the alkalinisation rates of both transporters were maximal at pH_i 6.5–6.6, and almost linearly decreased with the increase of pH_i, still being significantly active (about 20%) at pH_i 7.2.

Table 4 reports that the rate of H^+ flux (expressed as μM/s) through the Na^+/H^+ exchanger was significantly higher in the parental U-2 OS cell line and in

U-2OS cells transfected with vector alone (U-2/neo8 cells) in comparison with U-2/MDR117.1, U-2/MDR117.2 and U-2/DOXO-35 clones. Conversely, the values of Na^+-dependent HCO_3^-/Cl^- exchange activity were similar in the various cell lines. The relative contribution of the two transporters seems different in various clones, as shown by the ratio of the H^+ efflux rates, which increased in MDR-1 transfected cells in comparison to controls and even more in transfected cells after selection with doxorubicin. Preincubation of U-2/MDR117.1 and U-2/MDR117.2 cells for 30 min in the presence of 2 μM SDZ PSC 833 or 10 μM verapamil, inhibitors of P-glycoprotein, did not modify H^+ fluxes through the Na^+/H^+ exchanger at pH_i of 6.6 (in U-2/MDR117.1 cells: control 298 ± 70 μM/s, $n=7$, with SDZ PSC 833 278 ± 33 μM/s, $n=3$, with verapamil 258 ± 35 μM/s, $n=3$; in U-2/MDR117.2 cells: control 287 ± 45 μM/s, $n=5$, with SDZ PSC 833 264 ± 26 μM/s, $n=3$, with verapamil 257 ± 25 μM/s, $n=3$).

The rates of Na^+/H^+ exchange for vector-transfected U-2/neo-8 cells and the two MDR1-transfected clones were determined over a wide pH_i range, in order to characterise the kinetics of this exchanger with respect to intracellular $[H^+]$ (Fig. 7). Kinetic analysis showed that the Na^+/H^+ exchanger rates fitted a sigmoidal curve in all cell lines. Both the V_{max} and the K' values were significantly reduced in transfected cells in comparison with vector-transfected cells. It is noteworthy that U-2 OS cells exhibited a curve almost superimposed on that obtained with U-2/neo8 cells ($V_{max} = 540 \pm 20$ μM/s; $K' = 0.108 \pm 0.005$ μM^n; $n_{app} = 2.7$).

At least four isoforms of the Na^+/H^+ have been identified so far in human cells, NHE1, NHE2, NHE3 and NHE5 (for a review see [24]). By means of RT–PCR analysis, the presence of NHE1, NHE2 and NHE5 but not of NHE3 mRNAs was detected in all of U-2 OS variants (Fig. 8). The intensity of the GAPDH PCR product (control gene) was used for normalisation of the RT–PCR results, showing no significant variation in the expression of NHE1, NHE2 and NHE5 mRNAs among U-2 OS parental cell line and its MDR transfectants.

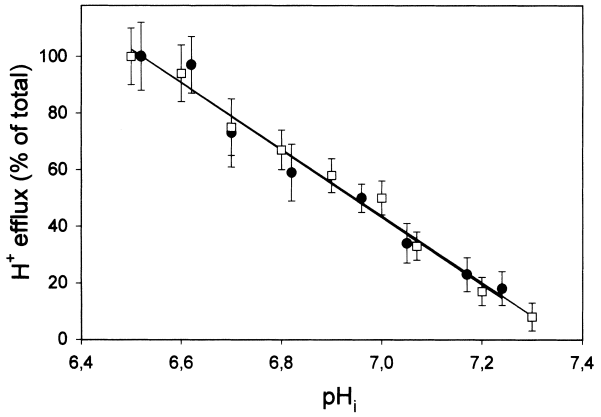

Fig. 6. H^+ efflux rate through the Na^+/H^+ exchanger and Na^+-dependent HCO_3^-/Cl^- exchanger as a function of pHi. BCECF-loaded U-2 OS cells were incubated in NMG-Cl saline solution in the presence of 0.6 μM nigericin, as shown in Fig. 4A, and pH_i was clamped at different pH_i by addition of 4.2 mg/ml BSA. Forty mM NaCl was added (●). Cells were treated as in Fig. 5B, 0.5 mM amiloride and 40 mM NaCl were added, followed by 20 mM $NaHCO_3$ (□). The initial rate of pH_i increase was determined after 1 min and data (mean ± S.D. of three experiments) are expressed as percent of total H^+ efflux. One hundred percent value was 472 ± 57 ($n=3$) and 85 ± 10 ($n=3$) μM/s for the Na^+/H^+ exchanger and Na^+-dependent HCO_3^-/Cl^- exchanger, respectively.

Table 4
H^+ fluxes through the Na^+/H^+ exchanger and Na^+-dependent HCO_3^-/Cl^- exchanger in the different U-2 OS clones

Cell line	H^+ flux (μM/s)		Ratio[a]
	Na^+/H^+ exchanger	Na^+-dependent HCO_3^-/Cl^- exchanger	
U-2 OS	456 ± 63 (11)	79 ± 28 (3)	0.17
U-2/neo8	427 ± 54 (4)	91 ± 14 (3)	0.21
U-2/MDR117.1	298 ± 70 (7)*	95 ± 13 (3)	0.32
U-2/MDR117.2	287 ± 45 (5)*	91 ± 11 (3)	0.32
U-2/DOXO-35	230 ± 26 (5)*	117 ± 5 (3)	0.51

The rates of H^+ efflux by the Na^+/H^+ exchanger and Na^+-dependent HCO_3^-/Cl^- exchanger were calculated from the initial rate of H^+ efflux measured over the first minute of pH_i recovery from pH_i 6.6 and expressed as H^+ flux, determined from ΔpH/min using the values of β_i of 57 mM/ΔpH. Experimental conditions as in Fig. 4A and 5A, respectively. Data are expressed as mean ± S.D. *Significant difference from U-2 OS cell line ($P < 0.05$).

[a]Na^+-dependent HCO_3^-/Cl^- exchanger rate over that through the Na^+/H^+ exchanger.

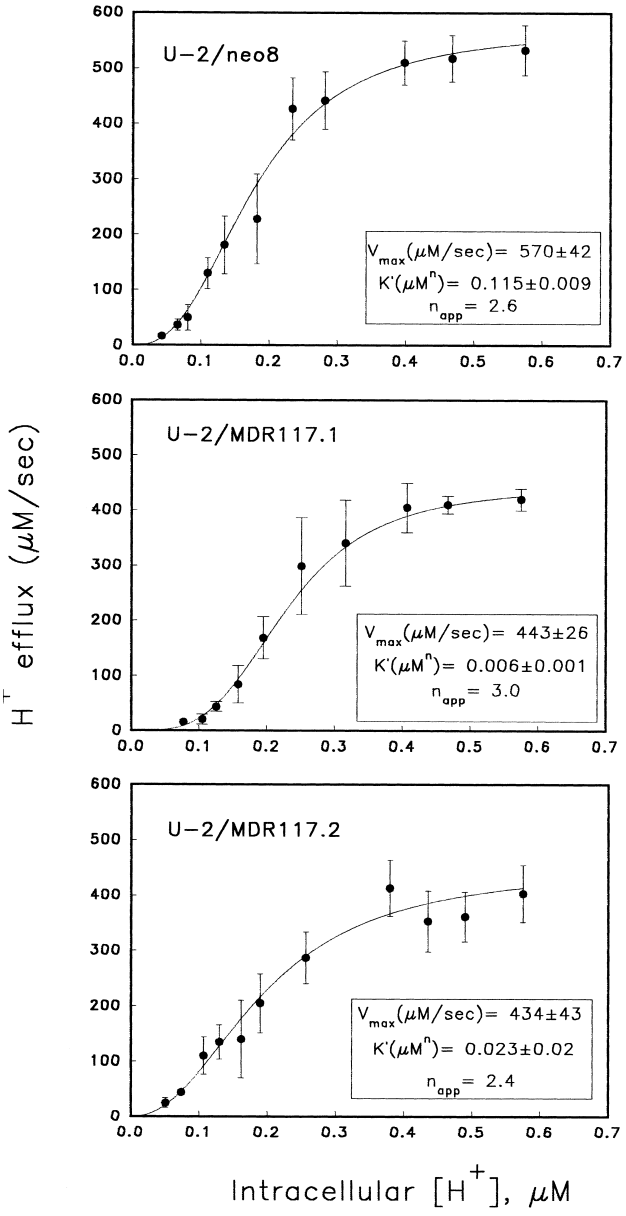

Fig. 7. H^+ efflux rate through the Na^+/H^+ exchanger as a function of intracellular [H^+] in vector- and P-glycoprotein-transfected U-2 OS clones. BCECF-loaded cells were incubated in NMG-Cl saline solution in the presence of 0.6 μM nigericin and pH_i was clamped at different pH_i by addition of 4.2 mg/ml BSA. Forty mM NaCl was added. Data were fitted by an allosteric kinetic model, as described in Section 2. Data are mean ± S.D. of 3–11 independent determinations.

4. Discussion

In the present study we report that the resting pH_i values of parental U-2 OS or vector-transfected U-2-neo8 cells and the *MDR-1* gene transfected clones selected in the absence or presence of doxorubicin were not significant different. The pH_i values of the various U-2 OS-derived clones were based on calibration with nigericin, a K^+/H^+ exchanger, assuming that the intracellular concentration of K^+ is similar. Because K^+ is the major intracellular cation, it is unlikely that it might be significantly different in the different U-2 OS-derived clones. Furthermore, both alkalinisation in HCO_3^--containing solutions and acidification in HCO_3^--free solutions were similar in all U-2 OS clones, clearly indicating the lack of H^+ transport perturbation associated with expression of P-glycoprotein. These results are in disagreement with those previously reported in LR73 Chinese hamster ovary fibroblasts transfected with *MDR-1*, which were shown to display a more alkaline resting cytosolic pH than non-transfected cells [10,25] and to undergo a quite extensive acidification during Cl^- substitution experiments [10]. The reason for this discrepancy might depend on several factors. A first difference might be due to the fact that our data were obtained in a population of detached cells, and not in single cells in monolayer. However, pH_i measurements from cell populations, which represent the average responses of several cells, although clearly less accurate, are certainly suitable for determination of changes of pH_i higher than 1.5 pH units, such as those reported in [10].

Another more substantial difference might be related to the level of P-glycoprotein expression, since it has previously reported that the value of resting pH_i raised when P-glycoprotein level was increased [10]. However, this possible explanation seems unlikely, since the level of P-glycoprotein expression was shown to be significantly higher in U-2/DOXO35 in comparison with U-2/MDR117.1 or U-2/MDR117.2 clones [11], whereas the pH_i values determined in the present study were similar.

Finally, an important distinction between our data and those previously reported might be related to the fact that parental and transfected U-2 OS clones lack a significant Na^+-independent HCO_3^-/Cl^- exchanger activity. This finding is in some way surprising, although similar results were previously reported also in U937 cells [26]. The analysis of other human osteosarcoma cell lines showing different differentiative features would be of help in order to clarify whether the lack of the Na^+-independent HCO_3^-/

Cl^- exchanger is a common or rather a sporadic characteristic of this neoplasm.

It is noteworthy that in LR73 Chinese hamster ovary fibroblasts, transfection with P-glycoprotein caused a complete inhibition of HCO_3^-/Cl^- exchanger activity, which should remove the excess base from the cytosol. The inhibition of HCO_3^-/Cl^- exchanger might help to explain the elevated pH_i reported in many MDR cells [25]. The pH_i maintenance and regulation was determined in various cell lines exhibiting MDR phenotype also by Belhuossine et al. [27] and by Litman et al. [28], with conflicting results. However, both these studies were performed in cells where P-glycoprotein expression was induced not by transfection, but by selection in doxorubicin or daunorubicin [27,28], which is known to alter cellular phenotype in a non-specific fashion. In conclusion, the results presented here indicate that a remarkable perturbation of pH_i does not occur in U-2 OS cells as a consequence of P-glycoprotein overexpression. This is in agreement with recent data, showing that transient expression of P-glycoprotein in HeLa cells had no remarkable effect on cellular pH [29].

We have then analysed the molecular mechanisms involved in the pH_i recovery from an acidic pulse in the parental U-2 OS cell line and revealed the presence of two distinct mechanisms: the first and most important is the amiloride-sensitive Na^+/H^+ exchanger, the second mechanism is the amiloride-insensitive, H_2DIDS-sensitive Na^+-dependent HCO_3^-/Cl^- exchanger. The contribution of Na^+/HCO_3^- cotransport to amiloride-insensitive alkalinisation was ruled out by complete inhibition observed after depletion of intracellular Cl^- and by inhibition by H_2DIDS, which was effective at concentrations higher than 0.1 mM. The relative rates of H^+ efflux through the two transporters were rather different, being that HCO_3^--dependent approximately one-sixth of the rate of the amiloride-sensitive mechanisms in U-2 OS parental cells. It is noteworthy that both pH_i regulatory mechanisms became quiescent at $pH_i > 7.2$ and that their pH_i dependence was superimposed. This result is in agreement with data previously reported in U937 human leukaemia cells [26], in barnacle muscle [30] and rat thymic lymphocytes [13]. Furthermore, the rate of H^+ efflux through the Na^+/H^+ exchanger, but not through the HCO_3^--dependent mechanism, was significantly reduced in U2-OS clones overexpressing P-glycoprotein, both in the absence and presence of doxorubicin selection. The contribution of the HCO_3^--dependent mechanism to overall H^+ efflux became more relevant in the two MDR clones selected with neomycin and maximal in the MDR clone selected with doxorubicin, where the activity of the Na^+/H^+ exchanger was significantly reduced (less than one-third and one-half, respectively, of the Na^+/H^+ exchanger activity).

The finding that the resting pH_i values were similar in control and MDR1-transfected clones, which exhibit a reduction of Na^+/H^+ exchanger activity, might be explained considering that the Na^+/H^+ exchanger activity is dependent on pH_i, significant at acidic pH_i (6.5–6.6), but almost negligible at the normal values of pH_i (7.0–7.2). Therefore, partial impairment of its activity might not necessarily lead to complete loss of pH_i regulation in the cells, unless a dramatically drop to acidic value occurs. Furthermore, it is possible that a significant compensatory H^+ efflux through the Na^+-dependent HCO_3^-/Cl^- exchanger or even through other H^+ transport mechanisms not considered in this study, i.e., the H^+-ATPase, might occur.

The rate data of H^+ efflux through the Na^+/H^+ exchanger as a function of intracellular $[H^+]$ from vector- and MDR1-transfected cells were analysed using non-linear regression to fit the data to the Hill equation, which provides a model for allosteric kinetics. This regression procedure permitted a careful determination of the kinetic parameters, properly weighting the experimental uncertainty of the experimental data [15]. Our data clearly show a sigmoidal relationship between the Na^+/H^+ exchange rate and the intracellular H^+ concentration in the three cell lines, indicating cooperativity in the activation of transport by intracellular H^+. Such pH_i dependence suggests the involvement of more than one H^+ binding site. This result is in agreement with data previously reported, showing the existence of a binding site(s) for the allosteric activation by internal H^+ (H^+ 'modifier' site) that is different from the H^+ transport site [31]. Whereas the values of n_{app} determined in vector- and P-glycoprotein-transfected U-2 OS cells were rather similar, both the V_{max} and K' values were significantly different. In particular, the V_{max} values were lower in cells expressing P-glycoprotein, and this might be achieved by reduction either in the maximal turnover rate of single transport-

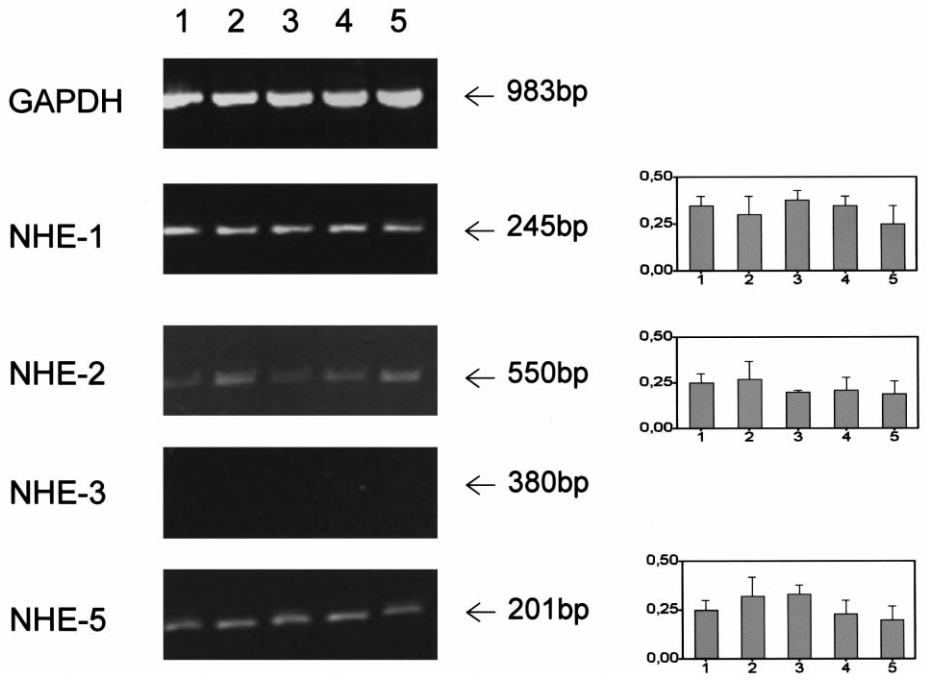

Fig. 8. RT–PCR analysis of the NHE isoforms in U-2 OS parental cells and MDR transfectants. Total RNA was reverse transcribed and amplified with specific primers as described in Section 2. Amplified products were analysed after 28 cycles by electrophoresis in 2% agarose gel. Agarose gels from a typical experiment are shown on the left; molecular sizes, in base pairs, are indicated on the right. For each NHE isoform, the intensity of the amplified product in the different U-2 OS clones was determined by using a dedicated image analysis system and normalized with the relative value of the GAPDH PCR product (control gene). Each bar plot on the right indicates the mean ± S.D. of the normalized values of NHE PCR product intensity from at least three experiments. The U 2-OS clones are indicated as follows: 1, U-2 OS; 2, U-2/neo8; 3, U-2/DOXO35; 4, U-2/MDR117.1; 5, U-2/MDR117.2.

ers or in the number of active Na^+/H^+ exchangers present in the plasma membrane.

By means of RT–PCR analysis, we detected remarkable mRNA levels of NHE1, NHE2 and NHE5 isoforms in U-2 OS-derived cell lines. The levels of the three isoform mRNAs were not significantly altered by transfection with *MDR1* gene, ruling out the possible complete down-regulation of expression of one NHE isoforms at mRNA level. However, from these mRNA results no conclusion on the protein level expression can be drawn. The lack of commercially available antibodies against the NHE isoforms, except for NHE1, makes difficult to assess the effect at protein expression level. It has to be also considered that subtle changes in protein levels, such as those expected from a 35–37% reduction in the activity, are likely to be hardly appreciated through a western blotting analysis.

Also, the K' values were significantly decreased in MDR-transfectants. In an allosteric model, K' represents a complex term comprised of the affinity constants of two or more binding sites, interaction factors and dissociation constants [16]. Thus, no simple physiological or kinetic interpretation of this parameter is possible. As previously described [23], K' estimates provide only an indication of the relative affinities of the Na^+/H^+ exchanger for intracellular $[H^+]$, which is clearly increased in MDR1-transfected cells in comparison to control. It is difficult to make a reasonable interpretation of the changes in the K' and V_{max} values of the Na^+/H^+ exchanger, because they represent the average values resulting from contribution of the three isoforms. Studies with cell lines co-expressing P-glycoprotein with NHE1 or NHE2 or NHE5 will help to define which NHE isoform is directly influenced by P-glycoprotein overexpression.

Previously reported data showed an increase in the activity of the Na^+/H^+ exchanger in MDR resistant cells relative to the drug-sensitive cells [6]. However, these results were obtained in cells where P-glycoprotein expression was induced by selection in doxorubicin, and not by transfection. The fact that the rate of the Na^+/H^+ exchanger in U-2 OS MDR transfectants further selected with doxorubicin was similar

to that detected in the absence of drug selection suggests that the kinetic alteration described here is likely to be strictly dependent on the P-glycoprotein expression, and not on drug selection. The results of the experiments in MDR-1 transfected cells in the presence of the P-glycoprotein inhibitors SDZ PSC 833 or verapamil suggest that the expression of a non-functional P-glycoprotein seems to be sufficient to affect the kinetics of the Na^+/H^+ exchanger. In this respect, it is interesting that Lee et al. [32] reported that the activation of the Cl^-/HCO_3^- exchanger by expression of the other ABC family member CFTR occurs also in the presence of the inhibitor glibenclamide, i.e., in the absence of its Cl^- conductive function. In particular, a mutational analysis pointed out the importance of nucleotide binding domain 2 (NBD2) of CFTR in the regulation of other ion channel or transport proteins [32]. Studies with U-2 OS cells transfected with P-glycoprotein mutated in the different functional domains would help to identify those possibly involved in the interaction with the Na^+/H^+ exchanger.

In conclusion, our findings indicate that, at least in osteosarcoma U-2 OS cells, the expression of P-glycoprotein induces a significant change in the kinetic parameters of the Na^+/H^+ exchanger, which can be appreciated at acidic pH_i. It can be proposed that this alteration might be the consequence of a direct or indirect interaction between the two transport mechanisms, although data indicating a direct interaction among P-glycoprotein and other transporters or membrane proteins are not yet available. It is possible that one of the NHE isoforms might directly associate with P-glycoprotein, or that other accessory proteins or regulatory cofactors, such as the NHE-regulatory factor or its human homologue ERB-50 [33,34], might mediate an indirect interaction.

Concerning the role of P-glycoprotein in tumour cells, it is of interest that the dominant mechanisms responsible for the regulation of pH_i under moderately acidic conditions, such as those occurring in the microenvironment of solid tumours, are the Na^+/H^+ exchanger and even more the Na^+-dependent HCO_3^-/Cl^- exchanger [35]. Both these transporters are active in the parental U-2 OS human osteosarcoma cells at acidic pH. However, H^+ efflux through the Na^+/H^+ exchanger is expected to be decreased in MDR1 transfected clones, leading to a reduction of H^+ released into the extracellular medium. It is possible that the reduced extracellular acidity might contribute to an impairment of the malignant potential observed in MDR transfectant cells, although it cannot be ruled out that overexpression of P-glycoprotein might result in the co-expression of other, still undefined genes, that are involved in the malignant behaviour.

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